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[1] Preparation of Monoclonal Antibodies: Strategies and Procedures

By G. GALFRÈ and C. MILSTEIN

I. Introduction

The derivation of cell lines capable of permanent production of specific antibody directed against a predefined immunogen was first reported in 1975.[1] The method was based on fusion between myeloma cells and spleen cells from suitably immunized animals. Spleen cells die in a short time under ordinary tissue culture conditions. Myeloma cells have been adapted to grow permanently in culture, and mutants were isolated that lacked the enzymes hypoxanthine guanine ribosyltransferase (azaguanine-resistant) or thymidine kinase (bromodeoxyuridine-resistant). Such mutants cannot grow in medium containing aminopterin and supplemented with hypoxanthine and thymidine (HAT medium) because they are unable to utilize the salvage pathway. Hybrids between such cells and spleen cells can be selected from the parental components as the only cells that actively multiply in HAT selective medium. From the growing hybrids, individual clones can be selected that secrete the desired antibodies. Such antibodies are therefore of monoclonal origin. The selected clones, like ordinary myeloma lines, can be maintained indefinitely.

This basic methodology has been used to prepare antibodies against a large variety of antigens. These include antibodies to haptens, small natural products with biological activity, such as neuropeptide and peptide hormones, enzymes and other proteins, polysaccharides, glycoproteins, lipopolysaccharides, histocompatibility antigens, differentiation antigens and other cell surface antigens, viruses, etc. The results justify the idea that the production of any antibody synthesized by the immunized animal can be immortalized by cell fusion methods.

Cell fusion is therefore a way of immortalizing cells expressing a transient differentiated function. The outcome of the fusion between a given cell line and a heterogeneous population of normal cells is affected by the phenotype of the particular cell line used. Fusions with myelomas result in a high frequency of antibody-secreting hybrids. On the other hand, other cell lines, for example, T cell lymphomas, are used for the immortalization of other differentiated properties, such as T cell functions. The derivation of such hybrids is based on the same general principles. Since the out-

[1] G. Köhler and C. Milstein, *Nature* (*London*) **256**, 495 (1975).

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come is not an antibody that can be used as a general reagent, it will not be discussed further.

The derivation of permanent lines of hybrid cells producing monoclonal antibodies (McAb) exhibiting certain desired properties presents widely different degrees of difficulty. Desired properties include not only specific recognition of an antigen; other no less critical properties are the fine specificity of the antibody, avidity and kinetic parameters important for radioimmunoassays, cytotoxic properties necessary for direct complement-dependent lysis, etc. When an animal is injected with a given antigen its usual response is the production of a highly heterogeneous population of antibodies directed against the immunogen. Among these many antibodies, some may have the desired properties but will be mixed with many others that will express alternative or undesirable properties. When McAb are prepared by the hybrid myeloma method, the collection of clones randomly derived represents a cross section of such a heterogeneous population. In addition the overall response of the individual animal can be strong or very weak, and this will be reflected in the proportion of hybrid clones producing the desired antibody within the total population of actively growing hybrids. These considerations are paramount in the preliminary estimation of the degree of difficulty that may be involved in the derivation of specific reagents. No less important is the fact that the characteristics of the McAb that will be derived will depend to a large extent on the way the whole experiment was originally designed. In this chapter we will attempt to provide guidelines for the derivation of specific McAb. For this we will draw on our own experience and will only occasionally refer to protocols and approaches that are not in use in our own laboratory. This is not because we consider our experience more valid than that of others, but, as in many complex operations, the final blend reflects the unique experience of the particular laboratory.

II. Materials for Tissue Culture[2]

A. Media

Tissue culture grade water is used throughout. This is usually deionized and double-distilled over glass.

For the preparation of McAb the most commonly used media are Dulbecco's Modified Eagle's Medium (DMM) and RPMI-1640. For practical reasons it is better to standardize with one medium. Alternative media are often required for cell lines from other laboratories. Whichever medium is chosen, particular attention must be devoted to its preparation. We will discuss here only the use of DMM, but the same general principles apply to any media. DMM is commercially available in different forms.

1 × DMM: The ready-to-use DMM (1 × DMM) can be bought as complete medium to which only pyruvate or glutamine and extra components are added before use, following the manufacturer's instructions (1 × DMM, Gibco Europe, Glasgow, Scotland, Cat. No. 196G; or Flow Laboratories, Irvine, Scotland, Cat. No. 12-334-54). Different batches may vary slightly. It is therefore advisable to buy batches in sufficient quantities to allow work for a reasonable period. The 1 × media will in general decay during storage; they are kept in the dark at 4°. For critical operations it is advisable to use media that are not more than 3 months old—especially when, as is the case with the 1 × medium, the exact date of preparation is not known.

10 × DMM: Ten times concentrate solutions (10 × DMM, Gibco Europe, Glasgow, Cat. No. 330-2501; or Flow Laboratories, Irvine, Cat. No. 14-330-49) are also available. About 4 liters of tissue culture grade water are autoclaved in a 6-liter glass flask and allowed to cool to room temperature. The 10 × medium and other components as specified by the manufacturer are then added. If necessary the volume is adjusted to approximately 5 liters with water.

Powder DMM: Prepared from dry powder (Gibco Laboratories, Grand Island, New York, Cat. No. 430-2100) following the manufacturer's instructions. This requires filter sterilizing units of 20-liter capacity or larger.

We find media prepared directly from powder to be the best, probably because they are usually used when fresher. The 1 × medium is almost as good but is much more expensive and requires more 4° storage space. We favor a supply of a few liters of 1 × DMM for comparison and for emergency cases. The medium prepared from the 10 × concentrate is generally not as good, and the batches are more variable. We use it only on well established lines when our production capacity from powder medium cannot cope with large-scale cultures. Concentrated medium is necessary for cloning in soft agar or agarose, and it is best to prepare 2 × medium from dry powder.

HAT medium

100 × HT: 136.1 mg of hypoxanthine (Sigma, Poole, Dorset, England, Cat. No. H9377) and 38.75 mg of thymidine (Sigma, Cat.

[2] A more detailed discussion on procedures for tissue culture can be found in J. Paul, "Cell and Tissue Culture," 5th ed., Churchill-Livingstone, Edinburgh and London, 1975; and W. B. Jakoby and I. H. Pastan, this series, Vol. 58.

No. T9250) are suspended in about 50 ml of water, and 0.1 *M* NaOH is added dropwise until dissolved. Adjust volume to 100 ml. Store at −20°. Thaw at 70° for 10–15 min.

50 × HT: Dilute 100 × HT with 1 volume of DMM. Filter sterilize and store in 25 ml aliquots at 4°.

1000 × aminopterin: Aminopterin (Sigma, Cat. No. A2255) 17.6 mg/100 ml. Proceed as for 100 × HT.

50 × HAT: 50 ml of 100 × HT, 5 ml of 1000 × aminopterin, and 45 ml of DMM. Filter, sterilize, and store in 25-ml aliquots at 4°.

1 × HT and 1 × HAT [20% fetal calf serum (FCS)]: 500 ml of DMM, 100 ml of FCS, 12 ml of 50 × HAT or 50 × HT, and antibiotics as required

B. Additives for Contamination Control

The most common tissue culture contaminants are bacteria, yeast, and fungi. To control them there is no substitute for a good, sterile technique. Bacterial contamination is not generally difficult to control with appropriate antibiotics. However, the routine inclusion of antibiotics in the medium leads to the selection of resistant bacteria. Sometimes these are slow growing and difficult to detect and become permanent and undesirable guests in the laboratory. A good compromise is to have penicillin and streptomycin (Gibco Europe, penicillin–streptomycin, 5000 units/ml, Cat. No. 507, used at a final dilution of 50 units/ml) routinely included in the medium. Gentamycin (Flow Laboratories, Cat. No. 16-762-45) is then reserved to control outbreaks of penicillin–streptomycin-resistant bacteria in important experiments. Gentamycin is said to be effective for mycoplasma infections. We have not found such infections a common problem. We have not ourselves found a satisfactory control for yeast and fungi. Contamination with yeast usually occurs in isolated cultures and does not spread. Fungal contamination is more difficult to confine. Spores quickly spread in the plates, out into the incubators, and eventually into the whole room. Particular attention must therefore be devoted to separating and eliminating the infected cultures as soon as possible. It is definitely worthwhile to prepare duplicates of important cultures in separate plates as soon as feasible.

C. Choice of Serum

Special care in the choice of serum is essential. Sera from different sources vary greatly, and each batch must be properly tested. Because of its low immunoglobulin content, FCS does not generally interfere with the assay of specific McAb. This is the most important reason for using FCS,

but not the only one; FCS seems also to give the highest efficiency in the preparation of hybrids. Heat inactivation is not usually necessary but may be required in specific cases. Because of the high price and extreme shortage of FCS, alternatives are being sought. Most parental myelomas were originally adapted to grow in medium supplemented with heat-inactivated horse serum, and early fusions were prepared with it. This was found to give unacceptable backgrounds when screening for certain antibody activities. Horse serum devoid of its γ-globulin fraction has been suggested as one alternative, but a wider search is required.

For the fusion, selection, and cloning steps, we recommend medium containing 20% FCS. As soon as a hybrid is selected we routinely shift from 20% to 10% FCS in the medium. When cells are well adapted we take them to 5% FCS. At concentrations lower than 5% FCS, cells grow more slowly, and this can be advantageous for routine maintenance.

If heat-inactivation is required it should be done carefully. Frozen bottles are thawed quickly in a 37° bath and left at 37° to warm up. They are then transferred to a 56° bath and left for 30–45 min, depending on the size of the container, with occasional mixing.

Testing of Serum Batches. Careful testing of the quality of serum batches is recommended in all cases. This is easily done by growth-efficiency tests. We routinely use a limiting dilution method as follows: From a logarithmic growing culture of any cell line, preferably a hybrid not yet growing vigorously, prepare four tubes containing 2000, 1000, 500, and 250 cells/ml. Dispense 150 μl of medium containing 20% FCS that is to be tested into the wells of rows 1–6 of a 96-well microtiter plate (Sterilin, Teddington, Middlesex, England, flat-bottom microtiter plates, Cat. No. M29ARTL). In rows 7–12 apply an equal volume of medium containing a control FCS for comparison. A multidispenser (e.g., Hamilton, Cat. No. PB600) fitted with a 10-ml plastic syringe is convenient. Apply 20 μl of each cell suspension into 24 consecutive wells. This is conveniently done with a multidispenser fitted with disposable 1-ml syringes. (Plastic syringes must be trimmed at the ring head to fit the dispenser or, better, the dispenser syringe-holder must be cut to allow the plastic syringe to snap in.) The plate is wrapped in cling film (e.g., Alcan Wrap) to reduce the risk of contamination and is incubated at 37° in a CO_2 humid incubator. After 3 days wells are examined for the presence of live cells, and after 7–10 days for active growth.

D. Equipment

The essential requirements are common to ordinary tissue culture laboratories and include 37° incubators with and without a controlled atmosphere of CO_2 and humidity. The CO_2 concentration should be adjusted to

give a steady pH of 7.2 to a sample of medium in an open container. Sterile work benches, inverted and ordinary microscopes preferably with phase contrast, water baths and/or hot blocks thermostatically controlled (e.g., Tecam Dry Block 08-3), centrifuge, liquid N_2 storage, plastic and glassware. Other items of equipment range from highly desirable to luxurious and are listed when recommended.

For long-term continuous culture and for mass culture of cells we strongly favor spinner vessels. These are enclosed glass vessels of 1–20-liter capacity with ports for delivery and removal of liquids and air and a Teflon-coated magnetic bar clear of the bottom of the vessel. A convenient arrangement for long-term cultures is shown in Fig. 1, in which a water-jacketed unit is used. These units are better than the non-water-jacketed type in terms of reliability of temperature control, but they are more cumbersome and therefore less convenient for short-term mass cultures. Components should be glass or Teflon as far as possible. Flexible tubing must be tissue culture grade (e.g., silicon rubber). When metal parts cannot be avoided, these must be of stainless steel 18/8 grade.

Mouth-pipetting is not recommended. We use a pipette-aid (Drummond Scientific Co., supplied by Bellco Glass Inc., Cat. No. 1225-80122) to which a flexible rubber tubing is attached. In this way long, as well as short, pipettes can comfortably be used.

III. Parental Cells

The choice and preparation of the two types of cells that are used as parents during fusion is of paramount importance. In particular the immune state of the animal from which the spleen is taken can make all the difference between success and failure. For the rest of this chapter we will discuss only procedures utilizing spleen cells. However, other lymphoid organs can be used, particularly lymph nodes. Indeed in specific cases this may be a better alternative if used in conjunction with certain immunization protocols.[3]

A. Immunization of Animals

The purity of the immunogen per se is irrelevant. It becomes important only if (*a*) impure material gives weaker specific responses; (*b*) the methods of assay do not distinguish between antibodies to the specific component and antibodies to the impurities. Some antigens are immunodominant

[3] D. Zagury, L. Phalente, J. Bernard, E. Hollande, and G. Buttin, *Eur. J. Immunol.* 9, 1 (1979).

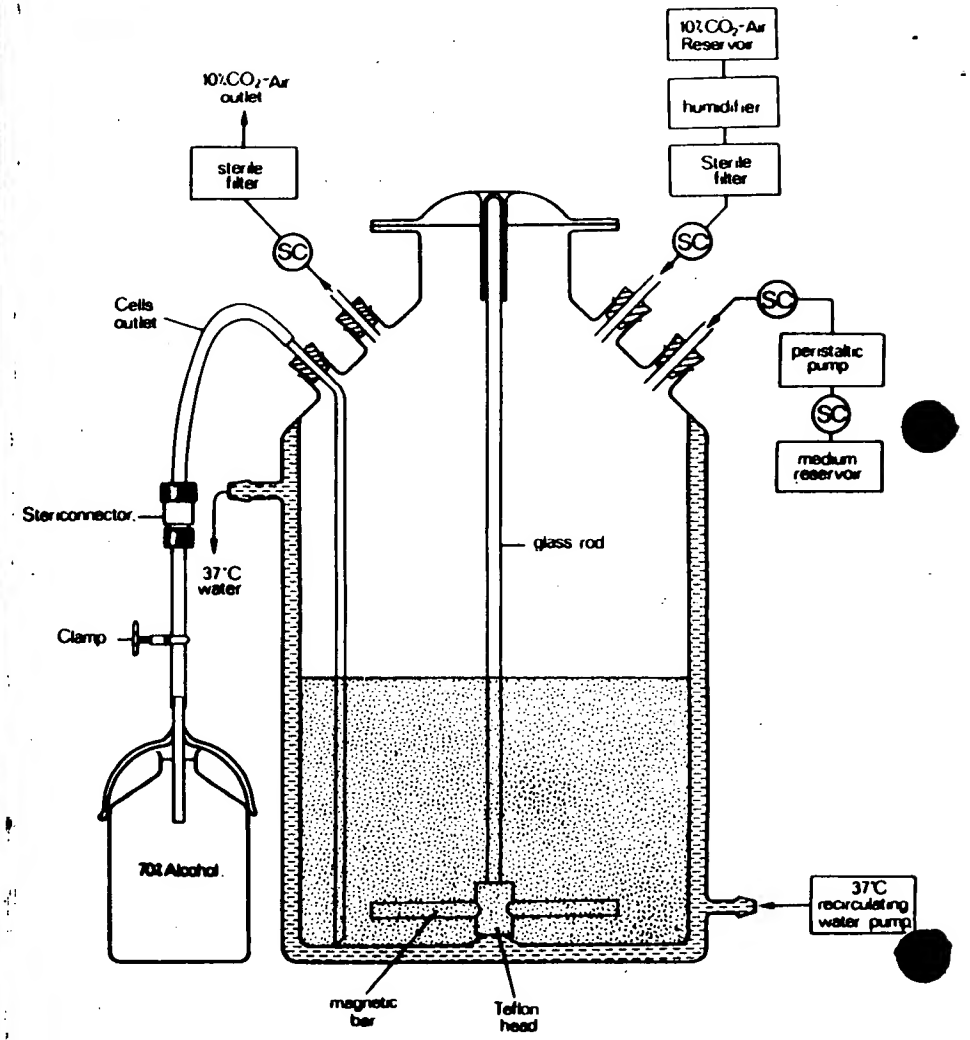

FIG. 1. Water-jacketed spinner vessel for continuous growth of cell lines. Simpler versions, with no water jacket, are used for short-term cultures and for 20-liter capacity (Cambridge Glassblowing, Crane Industrial Estate, or Wingent Engineering, Ltd., Cambridge, U.K.). SC are stericonnectors, size S 1/4 L.H. Engineering Co., Ltd., Stoke Poges, U.K.

and give strong immune responses even when present in only trace amounts. But the responsiveness of individual animals to the various chemical components of a mixture is rather variable, involving suppression as well as induction. There are so many factors to be taken into

consideration that exhaustive studies on the best immunization protocols are justified only in special cases. On the other hand, it is highly recommended that more than one immunization schedule should be tried, using several animals. Tests on different species and strains is a desirable practice. When no other information is available, note that the immunization protocol described below has been successful in many cases. In the final choice consideration should also be given to the species and strain of the parental myeloma. Interspecies hybrid lines are not suitable for production of antibodies in animals. If the animal chosen is of a strain different from that of the myeloma parent, the growth of tumor will require the use of F_1 hybrid animals. Other considerations being equal, the simplest animals to use are BALB/c mice and LOU rats.

Protocol. If the antigen is a soluble protein, a solution of about 1–5 mg/ml in saline is emulsified with an equal volume of Freund's complete adjuvant. This can be done by repeatedly squirting the suspension through the nozzle of a syringe. A total of about 0.3 or 0.6 ml is injected into multiple sites in mice and rats, respectively. The injections could be subcutaneous in at least three or four sites—for instance, in the back near the legs and the base of the tail. The treatment can be repeated at intervals of 3–5 weeks. About 10 days after each injection a drop of blood is taken by cutting the end of the tail of each animal, and this blood is used to test for the presence of specific antibodies. The animals giving the best antiserum are selected for fusion. After a rest period of a month or longer 0.2–0.4 ml of the protein solution (without Freund's adjuvant) is injected intravenously. The animals are sacrificed 3–4 days later, and the spleen cells are used as described in the fusion protocol.

The procedure can be speeded up by attempting a "blind" fusion using a primary immunized animal. In this case only an intravenous injection is performed 3–4 days before fusion.

B. Choice of Myelomas

The first point to consider is the species. Unless there are specific reasons against it, the myeloma should be of the same species as the immunized animal. This will permit easy development of tumors when hybrid myelomas have been derived. The choice between the rat and the mouse systems should be based on several considerations. The most important is the relative immune response to the antigen in question. If, after immunization of different rats and mice, individual animals show a better response, the myeloma parental cells should match the animal. If the responses are only marginally different, other considerations become important.

The rat system is better for the preparation of large amounts of antibody. Rats are considerably bigger than mice and just as easy to handle. Derivation of the hybrids with the rat lines has been found to be less straightforward than with the mouse lines, but with more experience the problems seem to disappear. On the other hand, the final recovery of positive clones from early hybrid cultures appears to be easier with rat lines. This may be because the percentage of growing hybrids expressing spleen immunoglobulins is 60% when mouse myeloma parental lines are used and over 90% with rat.[4] This is taken into consideration in the estimate of overall performance in Table I.

The next consideration is the chain composition of the myeloma. Hybrid myelomas codominantly express the immunoglobulin chains of both parental cells. If the myeloma line expresses both heavy and light chains of an immunoglobulin, the hybrid will express four chains. For convenience these are designated as follows: G and K are the heavy and light chains contributed by the parental myeloma; H and L are the respective chains (regardless of class or type) contributed by the spleen parental cells. Coexpression of chains from both parents *within* a single cell leads to the secretion of mixed molecular species. Thus, in addition to the parental types LHHL and KGGK, the hybrids will express immunoglobulin molecules of the type LHHK, KGGL, LGGL, and KHHK, regardless of class and type of chain. Moreover, mixed molecules containing both parental heavy chains of the type LHGK, KHGL, and all other permutations may also arise, but this depends on the class of the heavy chains. Although thorough investigations for all classes have not been carried out, the general rule seems to be that heavy chains of different subclasses, but not of different classes, can associate to form mixed molecules. For instance, $\gamma 1$ can combine with $\gamma 2a$ and $\gamma 2b$ but not with μ chains.

Hybrid myelomas of the type HLGK (i.e., expressing all four immunoglobulin chains) give rise with high frequency to mutant clones that no longer express one of the chains. This is not a random event, and the pattern of losses is shown in the diagram of Fig. 2. In Section IX, we describe the method for the derivation of segregants. It is much simpler to start with a myeloma that expresses only light chains. Such myelomas give rise directly to HLK hybrids (see diagram, Fig. 2). From here variants of the HL or HK type can be derived, but, particularly with the rat Y3 line, the frequency with which they arise is not so high. Using lines not expressing any myeloma chain (nonproducers), the hybrids will express only the antibody of the parental spleen. In Table I we give a subjective

[4] C. Milstein, M. R. Clark, G. Galfre, and A. C. Cuello, *in* "Immunology" (M. Fougereau, ed.), p. 17. Academic Press, New York, 1980.

TABLE I
PARENTAL MYELOMA LINES IN USE IN OUR LABORATORY

Name	Strain	Derived from	Immunoglobulin expression	Expected expression in hybrids	Estimated overall performance
Mouse lines					
P3-X63/Ag 8[a]	BALB/c	P3K[b]	MOPC 21[c] IgG_1 (κ)	HLGK[d]	++
NS1/1.Ag 4.1[e]	BALB/c	P3K	κ chains (nonsecreted)	HLK[e]	++
X63/Ag 8.653[f]	BALB/c	X63/Ag 8	None	HL	++
Sp2/O[g]	BALB/c	Hybrid Sp2[g]	None	HL	+
NSO/1[h]	BALB/c	NS1/1.Ag 4.1	None	HL	+++
Rat lines					
Y3-Ag 1.2.3[i]	Lou[a]	R210.RCY3[i]	S210 κ chain[m]	HLK	+++
YB2/3.0 Ag 20[n]	(Lou × AO)F_1	Hybrid YB2/3[o]	None	HL	+++

[a] Köhler and Milstein.[1]
[b] K. Horibata and A. W. Harris, *Exp. Cell Res.* **60**, 61 (1970).
[c] M. Potter, *Physiol. Rev.* **52**, 631 (1972).
[d] See also Fig. 2.
[e] Köhler *et al.*[32]
[f] J. F. Kearney, A. Radbruch, B. Liesegang, and K. Rajewsky, *J. Immunol.* **123**, 1548 (1979).
[g] M. Shulman, C. D. Wilde, and G. Köhler, *Nature (London)* **276**, 269 (1978).
[h] Sp2 is a hybrid myeloma prepared with X63/Ag 8 and a spleen from a BALB/c mouse immunized with sheep red cells.[1]
[i] This is a subline of NS1/1.Ag 4.1 that does not express the intracellular light chains (M. Clark, B. W. Wright and C. Milstein, unpublished data, 1980).
[j] Galfre *et al.*[6]
[k] Obtainable from H. Bazin, Ph.D., Experimental Immunology Unit, Bte UCL 3056, Clos Chapelle-Aux-Champs 30, 1200 Bruxelles, Belgium.
[l] R. G. H. Cotton and C. Milstein, *Nature (London)* **244**, 42 (1973).
[m] P. Queninjean, H. Bazin, A. Beckers, C. Beckers, J. F. Heremans, and C. Milstein, *Eur. J. Biochem.* **31**, 354 (1972).
[n] B. W. Wright and C. Milstein, unpublished data, 1980.
[o] YB2/3 is a hybrid myeloma prepared with Y3 cells and a spleen from an A0 rat immunized with human complement (see Lachman *et al.*[33]).

FIG. 2. Chain composition of hybrids and the derivation of segregants. H and L are the heavy and light chains contributed by the cells from the immune animal, and G and K are the myeloma heavy and light chains. 0 refers to no intracellular or secreted chains. The plus sign (+) indicates specific antibody. Dashed lines indicate uncommon or never observed.

assessment of the "performance" of each line. It is an attempt to estimate the relative chances of success in the derivation of specific clones taking a number of factors into consideration.

It follows that as a general rule the nonproducer myelomas are the best choice, especially as lines with good performance are now available. However, there are cases where the artificial combinations may be a desirable by-product. For instance, an HLK hybrid from which the variant clones HL and HK are prepared could provide an antibody (HL) and its ideal negative control (HK). For the preparation of standard reagents for commercial distribution, this may prove to be highly desirable. Other examples where such hybrids may be useful are in the preparation and testing of anti-idiotypic antibodies and when mixed molecules can be used for specific purposes.

Maintenance of Myeloma Cells. Whatever is the choice of myeloma, the most important factor for the successful derivation of hybrids is the way in which the myeloma culture has been maintained prior to fusion. The goal is logarithmic growth for as long as possible, certainly not less than a week before fusion. We strongly advise the use of spinner cultures as opposed to stationary suspension cultures. This may be an essential requirement when using the rat line Y3, which tends to stick to the walls of the culture vessel. Some workers advise the use of trypsin or other enzymes to detach the cells, but we have no experience of this procedure.

IV. Experimental Procedures

A scheme of the general procedures involved in the derivation of monoclonal antibodies is presented in Fig. 3. A number of well defined separate steps can be identified. These will be discussed individually under separate headings. However, it must be emphasized that this is by no means a rigid general protocol. Variations can be introduced at almost every step. Some variations, however, may affect more than a single step, and this should be carefully considered at the experimental design stage.

A. Preparation of Parental Cells for Fusion

1. Spleen Cells

Materials

FCS-DMM, 2.5%: 500 ml of DMM, 12 ml of FCS
CO_2 chamber: a 2-liter beaker containing Dry Ice covered with paper towels, with an aluminum foil lid
Alcohol, 70%: Prepare about 300 ml in a 500-ml beaker
Round-bottom plastic tubes (e.g., Sterilin 142AS), 10 ml
Pestle from a round-tip Teflon homogenizer to fit very loosely (1 mm clearance) the round-bottom plastic tubes
Sterile dissection instruments (forceps, scissors)

Procedure

1. Kill the animal by placing it in the CO_2 chamber for 1–2 min.
2. Dip it in 70% alcohol. Place it on a board in a sterile cabinet, and remove the spleen under sterile conditions.
3. Put the spleen in a petri dish containing about 5 ml of 2.5% FCS-DMM kept on ice, and wash gently.
4. Transfer the spleen to a 10-ml round-bottom tube, cutting it into three or four pieces. Add 5 ml of fresh 2.5% FCS-DMM.
5. With the Teflon pestle squash the pieces gently to make a cell suspension.
6. Allow the remaining clumps and pieces of connective tissue to sediment for about 3 min, then transfer the cell suspension to a 10-ml round-bottom plastic tube.
7. Fill the tube with 2.5% FCS-DMM and spin at room temperature for 7–10 min at 400 *g*. (During this interval start the preparation of myeloma cells as below.)
8. Resuspend pellet in about 10 ml of fresh medium and centrifuge as above.

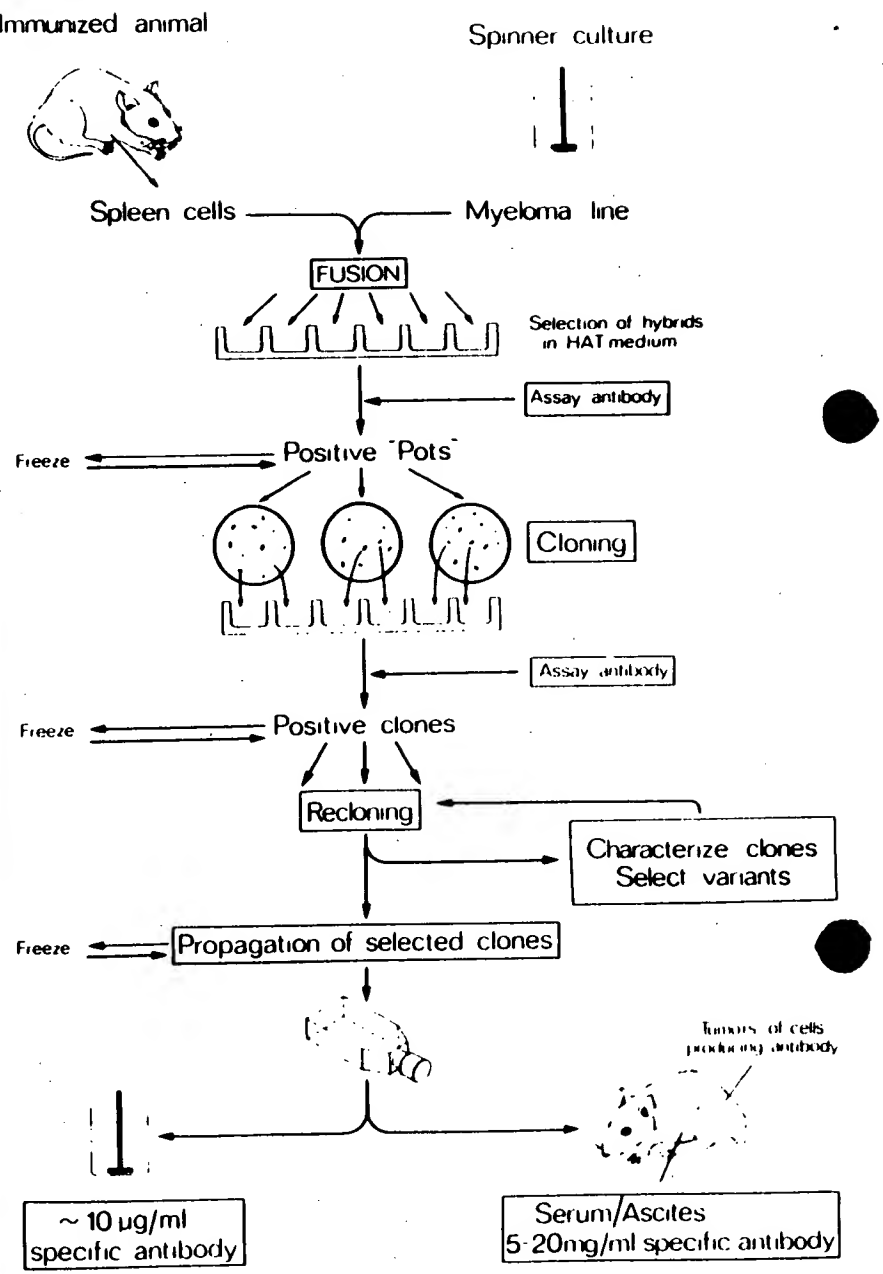

FIG. 3. Basic protocol for the derivation of monoclonal antibodies from hybrid myelomas.

9. Resuspend pellet in 10 ml of medium, and count the cells. This suspension can also be used as a feeder layer for the culture of the fused cells.

Viability, determined by phase-contrast microscopic examination (or Trypan blue exclusion test), should be higher than 80%.

2. *Myeloma Cells*

Enough (see below) myeloma cells from a culture in logarithmic growth are pelleted by centrifugation at room temperature for 10 min at 400 *g*. The pellet is resuspended in 10 ml of 2.5% FCS-DMM and counted.

B. Cell Fusion and Selection of Hybrids

Although the fusion and the initial selection of hybrids by growth in HAT medium are quite distinct stages, they are described together for convenience. We will describe in detail procedures that follow the general scheme of Fig. 3. There are ways in which certain stages can be bypassed. These will generally be dealt with under the heading Other Procedures (Section IV,B,3).

1. *Fusion of Cells in Suspension*[5,6]

Materials

Sterile conical tube, 50-ml (Falcon, Cat. No. 2070)
Sterile conical tube, 10-ml (Sterilin, Cat. No. 144AS)
Water bath, 40°
Hot block, 37°
Beaker, 200-ml
50% PEG: Polyethylene glycol (10 g), MW 1500 (BDH Chemicals Ltd., Poole, Dorset, England, Cat. No. 29575) is autoclaved in a 25-ml glass tube. While still liquid 10 ml of warm (37°) sterile DMM are added, and the solution is thoroughly mixed, inverting the tube. The pH is checked by the color of the phenol red and adjusted to around 7 by leaving the tube open in a sterile hood or by blowing 10% CO_2–air mixture into the tube.
Sterile pipettes, 1-, 10-, and 25-ml capacity
Linbro 24 wells plates (Flow Laboratories, Cat. No. 76-033-05)
DMM, 200 ml

[5] G. Galfrè, S. C. Howe, C. Milstein, G. W. Butcher, and J. C. Howard, *Nature (London)* 266, 550 (1977).
[6] G. Galfrè, C. Milstein, and B. W. Wright, *Nature (London)* 277, 131 (1979).

20% FCS–DMM and 2.5% FCS-DMM: 500 ml of DMM, 100 ml and 12 ml of FCS, respectively
HAT medium: 600 ml (see Section II,A)

Procedure

1. Parental cells are prepared as described above.
2. Mix 10^8 spleen cells and 10^7 (mouse) or 6×10^7 (rat) myeloma cells in a 50-ml conical tube; add DMM to a volume of 50 ml.
3. The cells are spun down at room temperature for 8 min at about 400 *g*.
4. The supernatant is removed with a Pasteur pipette connected to a vacuum line. Complete removal of the supernatant is essential to avoid dilution of PEG.
5. The pellet is broken by gently tapping the bottom of the tube. The tube is placed in a 200-ml beaker containing water at 40° and kept there during the fusion (steps 6–12). We do not consider it necessary to use a more cumbersome 37° water bath within the sterile cabinet.
6. Add 0.8 ml of 50% PEG prewarmed at 40° to the pellet using a 1-ml pipette, over a period of 1 min, continually stirring the cells with the pipette tip.
7. Stirring of the cells in 50% PEG is continued for a further 1.5–2 min. By then agglutination of cells must be evident.
8. With the same pipette, add 1 ml of DMM, taken from a tube containing 10 ml of DMM kept at 37° in the hot block, to the fusion mixture, continuously stirring as before, over a period of 1 min.
9. Repeat step 8.
10. Repeat step 8 twice, but add the medium in 30 sec.
11. Always with the same pipette and continuously stirring, add the rest of the 10 ml of DMM over a period of about 2 min.
12. With a 10-ml pipette add dropwise 12–13 ml of prewarmed DMM.
13. Spin down as in step 3.
14. Discard the supernatant, break the pellet by gently tapping the bottom of the tube, and resuspend in 49 ml of 20% FCS-DMM.
15. Distribute the fusion suspension in the 48 wells of two Linbro plates. These may contain a feeder layer of fibroblasts (see Section X).
16. Add a further 1 ml of 20% FCS-DMM. If a fibroblast feeder layer is not being used, add 10^5 spleen cells/ml (prepared as described in Section IV,A,1, step 9).
17. Incubate overnight at 37° in a CO_2 incubator.
18. With a Pasteur pipette connected to the vacuum line remove 1 ml

of culture medium from each cup without disturbing the cells that have settled in the bottom.

19. Feed the plate, adding 1 ml of HAT medium to each cup. Feeding with HAT medium is repeated for the next 2 or 3 days and after that once a week until vigorous growth of hybrids. This becomes evident under the microscope after day 10, but might take up to a month. At this stage the cultures become more yellow and are ready to be tested for antibody activity.
20. Duplicates of the growing hybrid cultures—either all or selected ones—are prepared and fed for a week with HT medium. Larger cultures can be prepared and frozen in liquid N_2. After a week in HT medium the cultures can be grown in the absence of HAT additives. Adaptation to lower concentration of serum can now be attempted.

2. *Filter Fusion*

The above procedure is not suitable for handling fewer than 4×10^7 spleen cells. For smaller numbers of cells we use a different protocol, essentially as described by Buttin *et al.*[1]

Materials

Filter fusion unit: We use the bottom half of a Millipore filtration set containing the mesh, the support, and the Teflon gasket. The top half holding the filter in position is replaced by a properly designed tube 3 cm long, made either of stainless steel or autoclavable plastic (see Fig. 4). The unit is fitted with a 25 mm 3.0 micropore size cellulose acetate filter (Millipore, S.A. Cat. No. SSWPO2500). The assembled unit is placed in an autoclavable centrifuge tube of appropriate size (e.g., M.S.E., Cat. No. 34411-166). The tube is closed, and the cap is held in position by a strip of autoclave tape.
Petri dishes: 3 cm diameter sterile (Sterilin, Cat. No. 301V); 4.5 cm diameter sterile (Sterilin, Cat. No. 302V)
Sterile forceps
Linbro 24 wells plate with feeder layer (see Section X)
Sterile pipettes: Pasteur, 10 and 25 ml capacity
Sterile tubes, 25 ml capacity
The following reagents as described above:
50% PEG, 2 ml
DMM, 5 ml

[1] G. Buttin, G. LeGuern, L. Phalente, E. C. C. Linn, L. Medrano, and P. A. Cazenave, *Curr. Top. Microbiol. Immunol.* **81**, 27 (1978).

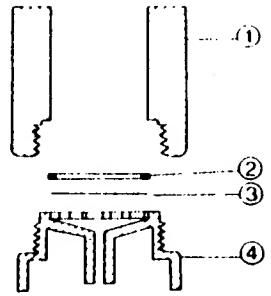

FIG. 4. Unit for filter fusions. The 3 cm-long tube (1) is of stainless steel 18/8 or Delrin (needs to be autoclaved and remachined a few times before use). The Teflon gasket (2) and filter support and mesh (4) are from a Swinnex-25 filter unit (Millipore S.A.); (3) is a cellulose acetate filter.

2.5% FCS-DMM, 20 ml
20% FCS-DMM, 50 ml
HAT medium

Procedure

1. Parental cells are prepared as described in Section IV,A.
2. Mix 10^6 spleen cells and 10^5 (mouse) or 6×10^5 (rat) myeloma cells and dilute to a total volume of 4 ml with DMM.
3. Open the tube containing the filter fusion unit and transfer the cell mixture to the unit.
4. Close the centrifuge tube and spin for 5 min at 400 *g* at room temperature.
5. Add 1.5 ml of warm (40°) 50% PEG to a 3-cm diameter petri dish.
6. After centrifugation open the centrifuge tube and, using sterile forceps, place the filter fusion unit in an open sterile petri dish (4.5 cm in diameter). No medium must be present on the filter.
7. Carefully dismantle the unit and remove the filter with sterile forceps.
8. Place the filter on top of the 50% PEG (step 5) with the cell layer facing down. Avoid bubbles under the filter. Incubate 1–3 min.
9. In the meantime transfer 5 ml of 20% FCS-DMM to a 4.5 cm diameter petri dish.
10. Remove the filter from the 50% PEG and place, cells facing down, in the 20% FCS-DMM.
11. Incubate overnight at 37° in a CO_2 incubator.
12. During the incubation most of the cells will detach from the filter

and settle on the bottom of the petri dish. Lift the filter with sterile forceps and wash the remaining adhering cells into the petri dish, using a Pasteur pipette and medium from the dish.

13. Using the same pipette transfer all the cells to a tube and add 20 ml of 20% FCS-DMM.
14. Distribute the cell suspension in the 24 wells of the previously prepared Linbro plate containing a feeder layer. Add 1 ml of HAT per well.
15. Incubate at 37° in a CO_2 incubator and proceed as described in Section IV,B,1 from step 18 on. Vigorous growth of hybrids will usually become discernible under the microscope after 2 weeks.

3. Other Procedures

Variations to the fusion protocol are described by several authors. The most important involve the addition of dimethyl sulfoxide to the polyethylene glycol,[8] changes in the concentration and molecular weight of polyethylene glycol as well as time of treatment,[9,10] centrifugation of cells on flat surfaces,[11] and variations in the ratio of spleen to myeloma cells. The choice of protocol does not seem to be critical, as all of them have been used successfully.

After fusion it is not necessary to fractionate the cell suspension as described in Section IV,B,1, step 15 on. Alternative procedures range from fractionation into a much greater number of microcultures, and in 200-μl well plates, to direct cloning onto semisolid agar.

4. Controls

Failure to grow hybrids after HAT selection is not uncommon on the introduction of the technique to a laboratory. This can be due to many reasons, but the first to be considered is the correct maintenance of the parental cells as discussed in Section III,B. Overgrown cultures will not recover in a few days, and frozen cultures are likely to take 2 weeks before they are in a suitable state for fusion. Poor media or accidental contamination with toxic substances is the second most common source of failure. The reagents and equipment used in the fusion and selection stages should be quality-controlled. For instance, the HAT medium should be controlled by growing established hybrids at low dilutions.

[8] T. H. Norwood, C. J. Zeigler, and G. M. Martin, *Som. Cell Gen.* 2, 263 (1976).
[9] R. L. Davidson, K. A. O'Malley, and T. B. Wheeler, *Som. Cell Gen.* 2, 271 (1976).
[10] V. L. Gefter, D. H. Margulies, and M. D. Scharff, *Som. Cell Gen.* 3, 231 (1977).
[11] K. A. O'Malley and R. L. Davidson, *Som. Cell Gen.* 3, 441 (1977).

Polyethylene glycol damages cells: overtreatment results in excessive cell death, undertreatment in insufficient fusion. The simplest check consists of careful examination of cells the day after fusion, before addition of HAT. The parental myeloma cells should show signs of growth. This can be most carefully controlled by limiting dilution analysis, comparing the growth in the absence of HAT of those cells treated and not treated with PEG.

Assays

The choice of assay used during the screening stages, to detect and clone the hybrid secreting the desired antibody, is of the utmost importance and should be given the greatest attention. Over the years immunologists have developed an enormous variety of ways of detecting the presence of antibodies, ranging from precipitation reactions and radioimmunoassays to assays based on the biological activities of the recognized antigens. Extensive reviews of such methods are to be found in other articles in this volume, in the "Handbook of Experimental Immunology,"[12] etc. But not all such assays are directly applicable to monoclonal antibodies. This is for two main reasons. First, the concentration of antibody in the tissue culture supernatant is usually much lower than that of a hyperimmune serum, and, second, traditional immunoassays often rely on the polyvalent recognition of antigens typically obtained with polyclonal antisera. Taking these factors into consideration, it is usually possible to adapt any immunoassay to detect monoclonal antibody in the supernatant of hybrid cultures. There are two general ways to detect the presence of antibody-secreting hybrids. The first utilizes the spent medium of growing cultures: the second directly detects the presence of antibody in the microenvironment of isolated cells or clones of cells grown in a semisolid medium.

V. Detection of Antibody in the Spent Medium

No method guarantees detection of all the clones secreting specific antibodies. For instance, not all immunoglobulin classes can fix complement, and therefore not all are detectable by direct lytic assay. This can be overcome by the addition of a second developing antibody (antiglobulin antibody, indirect lysis). However, the ratios of both antibodies are critical, and excess of either can inhibit lysis. The multiplicity of classes and subclasses makes it difficult to choose conditions that will ensure lysis for

[12] "Handbook of Experimental Immunology" (D. M. Weir, ed.), 3rd ed. Blackwell, Oxford, 1978.

all of them. Indirect binding assays are more generally used for their simplicity, accuracy, versatility and ability to detect the largest proportion of antibody-secreting clones. But even they are limited by the specificity of the second antibody and by the number of binding sites.

A. Binding Assays

The insoluble antigen and the antibody in the culture fluid are allowed to react. The free antibody is washed away. The amount of monoclonal antibody bound is measured directly (direct binding assay) or by binding of a second, labeled antibody capable of recognizing the first (indirect binding assay). This second antibody can be labeled in several ways. We will describe two of the most commonly used. Others include, for instance, enzyme-linked derivatives,[13,14] or the use of protein A as an alternative to the second antibody.[15] It should be remembered that protein A does not bind to some classes (notably IgM) or even to some IgG subclasses of mouse and rat antibodies.

1. Insolubilization of Antigen

Antigens are often naturally insoluble (e.g., cell surface antigens). Others need to be rendered insoluble, and this can be conveniently done by attachment to plastic, e.g., microtiter plates (as described below) or polystyrene balls.[16] If antigens are small molecules, like haptens, they can be conjugated with proteins as a preliminary step.

Materials

Phosphate buffered saline (PBS): NaCl, 8.0 g/liter; KCl, 0.2 g/liter; Na_2HPO_4, 1.15 g/liter; KH_2PO_4, 0.2 g/liter
Protein antigen solution: 20–100 μg/ml in PBS containing 5 m*M* EDTA, 0.1% azide
BSA–PBS: 10% BSA in PBS (w/v)
Microtiter plates: flexible polyvinyl chloride microtitration plates, 96 round U wells (Cooke microtitre plate, Cat. No. 1-220-24)

Procedure

1. Dispense 50 μl of protein antigen solution in each well of a microtiter plate except for those that are to be used as controls.
2. Incubate at 4° overnight.

[13] S. Avrameas, *Int. Rev. Cytol.* 27, 349 (1970).
[14] E. Engvall and P. Perlman, *Immunochemistry* 8, 871 (1971).
[15] S. Jonsson and G. Kronvall, *Eur. J. Immunol.* 4, 29 (1974).
[16] B. R. Ziola, M. T. Matikainen, and A. Salmi, *J. Immunol. Methods* 17, 309 (1977).

3. Empty the wells. Often the same solution can be used two or three times for coating plates, but it needs to be tested.
4. Fill the plate with BSA–PBS and incubate at room temperature for 3–4 hr. At this stage it can be stored for 1 week at 4°.
5. Remove the BSA–PBS.

2. Preparation of Iodine-Labeled Second Antibody

Materials

PBS: see above
Sephadex G-50 fine: preswollen in PBS
Chloramine-T: 2 mg/ml in 0.3 *M* sodium phosphate buffer, pH 7.3, prepared *fresh* before use.
Tyrosine solution: saturated solution of tyrosine in H_2O
1% BSA–PBS: 10% BSA (w/v) in PBS, adjusted to pH 7.3
Protein solution: about 1 mg/ml in PBS
^{125}I-labeled solution: sodium iodide pH 7–11, 13–17 mCi per microgram of iodine (Radiochemical Centre, Amersham, England, Cat. No. IMS.30)
Disposable pipette, 5-ml (Falcon pipette 7543)
Glass wool, Parafilm, glass test tube
Hamilton syringe, 50-μl
Several Pasteur pipettes

Procedure

1. Cut the 5-ml disposable pipette a few centimeters above the gradation, plug the pipette tip with a small amount of glass wool, and place in a stand.
2. Fill to the 5-cm mark with Sephadex G-50 fine in PBS.
3. Run 3 or 4 ml of 1% BSA–PBS through the column.
4. Wash the column thoroughly with several milliliters of PBS, leaving about 0.5 ml above the Sephadex.
5. Seal both ends of the column with Parafilm until ready for use.
6. Using 50-μl Hamilton syringe, transfer to the glass tube 10 μl of ^{125}I-labeled solution. Add 10 μl of chloramine-T and, quickly, 20 μl of protein solution.
7. Mix well and incubate for 15 sec–2 min (varies from protein to protein). Add 25 μl of tyrosine, 50 μl of 1% BSA–PBS, and 200 μl of PBS; mix.
8. Using a Pasteur pipette, carefully apply the mixture to the column prepared in steps 1–6.
9. Load three times in succession 0.2 ml and, finally, 0.5 ml of PBS.

10. Discard effluent.
11. Load 1.5 ml of PBS.
12. Collect effluent containing the labeled protein. The column can be washed with PBS and used several times.
13. Dilute 10 μl of labeled protein solution in 1 ml of PBS and count 10 μl of this dilution in a gamma counter. In a standard preparation about 5×10^5 cpm/μl of column eluate would be expected.
14. Store at −20° in small aliquots and use within 1–2 months.

3. *Indirect Binding Assay*[17,18]

Materials

PBS–10% FCS: PBS containing 10% FCS (or 1% BSA) and 0.1% NaN_3. Animal serum other than FCS can be used if it does not interfere with the assay.

^{125}I-labeled second antibody: prepared as above, adjusted to about 5×10^4 cpm/50 μl.

Microtiter plates: flexible polyvinyl chloride microtitration plates, 96 round U-wells (Cooke microtitre plate, Cat. No. 1-220-24)

Rotary plate shaker (optional): microshaker (Dynatech, Microtitre)

Centrifuge plate carrier: to spin plates in a refrigerated centrifuge when insoluble antigen (e.g., cells) not bound to the plates is used.

Hot wire cutter: a device formed by a rigid base (30 cm × 15 cm × 1 cm) with a tungsten wire across the middle of the base, kept stretched by a spring at a height of 8 mm from the base. An electric current from a variable rheostat is used to heat the wire to a proper temperature to cut the wells from the rest of the microtiter plate. This is done by sliding the plate along the base and slicing away the top part. Adhesive paper is stuck on the bottom of all the wells before they are cut out with the hot wire.

Multidispenser: multichannel reagent dispenser (Cooke Engineering Co.; available from Gibco-Europe, Cat. No. AM58)

Procedure

1. Antigen-coated microtiter plates are prepared as described in Section V,A,1. Alternatively, if cells or other particles are used as antigen, they are suspended in PBS–10% FCS. Apply to each well 50 μl of cell suspension containing between 5×10^5 and 6×10^6 cells.

[17] A. F. Williams, *Contemp. Top. Mol. Immunol.* 6, 83 (1977).
[18] L. A. Herzenberg and L. A. Herzenberg, *in* "Handbook of Experimental Immunology" (D. M. Weir, ed.), 3rd ed., Chapter 12.22. Blackwell, Oxford, 1978.

2. Add 50 μl of spent culture medium that is to be tested (first antibody) to each well. A negative control must be included, using tissue culture medium. A positive control containing dilutions of the serum of the immune animals, or other antibodies previously obtained, is desirable.
3. Mix contents of wells (about 10 sec if using a rotary plate shaker).
4. Cover the plate and incubate at 4° for 45–60 min.
5. Wash the plate. (*a*) If antigen-coated plates are used, fill each well with 150 μl of PBS–10% FCS (a multidispenser can be used) and empty by inverting and vigorously shaking the plate over a sink (or a 1-liter beaker if radioactive material is to be discarded separately). Repeat the cycle twice. (*b*) To wash the wells containing cells, spin the plate 5 min at 400 *g* at 4°. Remove medium by suction. Shake the plate for 10 sec in the rotary shaker. Add 200 μl of PBS–10% FCS to each well. Repeat the whole cycle, spin as above, and remove medium by suction.
6. Add 50 μl of radioactive second antibody.
7. Cover the plate and shake for about 10 sec in the shaker.
8. Incubate at 4° for 45–60 min.
9. Wash plate as above, adding at least one extra cycle.
10. Dry the plate for 30 min in a 37° oven and cut the wells with a hot wire. Care should be taken to keep the wells attached to adhesive paper to keep them in order.
11. Using forceps, transfer each well into clean, labeled counting tubes. Count in a gamma counter.

An alternative possibility after step 9 is to add 50 μl of PBS–10% FCS to each well. The pellets are resuspended by shaking the plate; they are then transferred to clean tubes to be counted. If the antigen is immobilized on the plate, 50 μl of 1 *N* NaOH can be used to solubilize the material.

4. *Fluoresceinated Second Antibody*

This reagent is easily adaptable to qualitative screening, especially in combination with fluorescent microscopy[19] and cytofluorometry.[20]

Materials

Buffer: NaCl, 1.5 g/liter; Na_2CO_3, 1.95 g/liter; $NaHCO_3$, 2.66 g/liter; pH 9.3

[19] G. D. Johnson, E. J. Holbrow, and J. Dorling *in* "Handbook of Experimental Immunology" (D. M. Weir, ed.), 3rd ed., Chapter 15.16. Blackwell, Oxford, 1978.
[20] L. A. Herzenberg and L. A. Herzenberg, *in* "Handbook of Experimental Immunology" (D. M. Weir, ed.), 3rd ed., Chapter 22.1. Blackwell, Oxford, 1978.

FITC solution: fluoresceinisothiocyanate (isomer I from Sigma, Cat. No. F7250), 1 mg/ml in buffer, pH 9.3
Protein solution: about 1 mg/ml in buffer pH 9.3
Sephadex G-50 column prepared exactly as described in the protocol for ^{125}I-labeling (steps 1–6) and equilibrated with PBS

Procedure

1. Add 0.3 ml of FITC solution to 1 ml of protein solution and incubate at room temperature for 3 hr.
2. Load onto the Sephadex G-50 column previously prepared.
3. Load three times in succession 0.2 ml and, finally, 0.5 ml of PBS.
4. Discard column effluent.
5. Load 1.5 ml of PBS.
6. Collect five fractions of about 0.3 ml.
7. Measure the optical density at 280 nm and at 495 nm. Pool the fractions, giving a ratio OD_{280}: OD_{495} of about 1. (Alternatively the fraction containing fluorescent protein can be identified under an ultraviolet light.)
8. Dilute with 1 volume of 10% BSA–PBS (0.1% NaN_3) and store at 4° or frozen in aliquots at −20°.
9. Dilute as appropriate before use.

5. *Preparation of Internally Labeled Antibody*

Unlike ordinary antibodies, monoclonal antibodies can be easily labeled internally at high specific activity, using radioactive amino acid precursors. The choice of these is based on the efficiency of incorporation of labeled amino acids into secreted immunoglobulin in culture conditions (Table II). We normally use radioactive lysine. Although the incorporation per amino acid residue is higher for arginine and phenylalanine, the number of lysine residues is usually higher. The more commonly used leucine is only about half as efficient as lysine. We reserve [^{35}S]Met or [^{35}S]Cys labeling for special uses. We routinely use [^{3}H]Lys for quantitative binding studies[6] and for immunocytochemistry.[21] For the chemical characterization of the monoclonal antibody (Section IX) it is simpler to use [^{14}C]Lys.

Materials

−Lys DMM: DMM without L-lysine (Gibco Bio-Cult)
Dialyzed FCS: fetal calf serum is dialyzed against double-distilled water. After dialysis, add 1/9th volume of 10 times balanced saline solution.
[^{3}H]Lys or [^{14}C]Lys: L-[4,5-^{3}H]lysine monohydrochloride, 5 mCi in 5 ml (The Radiochemical Centre, Amersham, England, Cat. No. TRK.520), or L-[U-^{14}C]lysine monohydrochloride, 250 μCi in 5 ml (Cat. No. CFB.69).
Incorporation medium: −Lys DMM, 9 ml; [^{14}C]Lys, 1 ml; dialyzed FCS, 0.5 ml or −Lys DMM, 2.5 ml; [^{3}H]Lys, 1.8 ml; 10 times balanced saline solution, 0.2 ml; dialyzed FCS, 0.5 ml

Procedure. About 2 × 10^6 cells from an exponentially growing culture are centrifuged, resuspended in −Lys DMM and pelleted by centrifugation. They are resuspended in 1 ml of incorporation medium and incubated at 37° in a water-saturated CO_2 incubator. Radioactive supernatant can be collected after 16–20 hr of incubation. Alternatively, after 8 hr of incubation a further 2 × 10^6 cells are washed as above and the pellet is

[6] A. C. Cuello, C. Milstein, and J. V. Priestley, *Brain Res. Bull.* 5, 5 (1980).

TABLE II
EFFICIENCY OF INCORPORATION OF DIFFERENT AMINO ACIDS INTO MYELOMA PROTEIN MOPC 21 UNDER TISSUE CULTURE CONDITIONS[a,b]

Amino acid	Radioactivity[c] (%)	Radioactivity residues[d]
Lys	20.1	3.2
His	1.6	0.6
Arg	15.5	5.5
Asp	0.6	0.06
Thr	11.2	1.1
Ser	4.2	0.3
Glu	1.3	0.1
Pro	7.6	0.8
Gly	3.6	0.5
Ala	0.5	0.1
Val	4.0	0.4
Met	0.9	0.4
Ile	3.2	1.0
Leu	8.2	1.4
Phe	17.3	4.1

[a] Unpublished data of J. Svasti and C. Milstein (1972).
[b] For this experiment the αα mixture was used and incorporation was measured after total hydrolysis of the purified IgG.
[c] Recovered after total hydrolysis.
[d] Refers to the number of moles of each residue per mole of protein after total hydrolysis.

added to the radioactive culture. The supernatant is collected after a further 10–12 hr of incubation.

For quantitative binding and immunocytochemical applications, purification is required to reduce the radioactive background. Often extensive dialysis is sufficient.[6] In other cases more extensive purification is required.[22]

6. *Inhibition of Direct Binding*

Among the quantitative binding studies, internally labeled monoclonal antibodies are particularly valuable to recognize other monoclonal antibodies with similar specificity. This is important for mapping antigenic determinants and detecting possible redundancy during the screening of hybrid cultures.[23,24] For these purposes we measure the inhibition of the direct binding of a labeled monoclonal antibody by the supernatants of the hybrids under study. The procedure is essentially as described in Section V,A,3 with the following modifications: (*a*) omission of step 5; (*b*) in step 6, the use of internally labeled monoclonal antibody instead of radioactive second antibody; (*c*) in step 11, transfer of each well to counting tubes and addition of 2 ml of Aquasol-2 (New England Nuclear, Cat. No. NEF-952) before counting. Quantitative inhibition studies require adequate titration of reagents.

Appropriate monoclonal anti-immunoglobulin antibodies can also be internally labeled and used as second antibody for indirect binding assays.

B. Hemagglutination Assays[25]

These assays are based on the ability of an antibody to agglutinate red cells carrying the specific antigen. They have all the advantages in terms of extreme simplicity, speed, and direct visual reading of results. The disadvantages are the inhibitory effects due to excess antibody (prozone effects) and quantitative inaccuracy. In practice these assays often fail to detect a number of antibody-secreting clones.

Inhibition of hemagglutination is a very simple way in which to define the specificity of the antibodies. This is done by simply adding excess antigen and antigen analogs to an appropriate dilution of hybrid supernatants before the addition of red blood cells.

[22] P. J. Lachmann, R. G. Oldroyd, C. Milstein, and B. W. Wright, *Immunology* **8**, 503 (1980).
[23] T. Springer, G. Galfre, D. Secher, and C. Milstein, *Eur. J. Immunol.* **8**, 539 (1978).
[24] J. C. Howard, G. W. Butcher, G. Galfre, C. Milstein, and C. P. Milstein, *Immunol. Rev.* **47**, 139 (1979).
[25] R. R. A. Coombs, *in* "Immunoassays for the 80s" (A. Voller, ed.), MTP Press, London, 1980.

1. *Attachment of Protein Antigens to Red Cells*

Materials

Red blood cells (RBC), usually from sheep
Saline: 0.9% NaCl in distilled water
$CrCl_3$ solution: 0.5 mg of $CrCl_3$ per milliliter in saline adjusted to about pH 5 by addition of NaOH, taking care to avoid the formation of any precipitate.
Protein antigen: about 1 mg/ml in saline. (Not PBS: phosphate inhibits $CrCl_3$ coupling.)
PBS, pH 7.2

Procedure

1. Wash the RBC three or four times in saline.
2. In a round-bottom tube containing 1 volume of packed RBC, add 1 volume of $CrCl_3$ solution and 1 volume of protein antigen solution. The two solutions should be added simultaneously, using two pipettes.
3. Immediately resuspend the cells by inverting the tube several times; continue this for 2 min.
4. Add at least 10 volumes of PBS; mix by inversion, and spin down at 1000 *g* for 5 min.
5. Repeat the wash three times and resuspend the coated RBS in PBS. Sterile coated RBC can be stored for several weeks at 4°.

2. *Direct Hemagglutination*

In each well of a microtiter plate (round-bottom U-wells) dispense 25 μl of RBC–PBS (1 : 16, v/v). Add 25 μl of supernatant to be tested and mix well using a plate shaker. Incubate at room temperature for 2 hr. Agglutinated RBC fail to settle as a tight pellet. The plate can be photographed. For a more accurate reading, the pellet of each well is carefully transferred onto a microscope slide. Microscopic examination can detect very weak agglutination.

3. *Indirect Hemagglutination*

At the end of the direct agglutination test it is possible to add a titrated amount of anti-immunoglobulin to each well. The pellets are then resuspended and allowed to settle for a further 2 hr. After this period, results can be recorded as above. Better, but more time-consuming, is to remove the first antibody before the addition of the second. The second antibody must be tested before use. It must not agglutinate coated RBC in the absence of the first antibody at the concentration used in the final test.

C. Lytic Assays

These assays are based on lysis of cells by antibody and complement. The extent of cell lysis can be measured in several ways. One is the release of ^{51}Cr incorporated into target cells carrying the desired antigen. This and other related methods are particularly applicable to cell surface antigens.[26,27] We will describe another method that is based on visual observation of lysis of red cells.[28] This is of general application. Soluble antigens can be attached to the red cell surface as is done for hemagglutination assays.

Spot Tests

Materials

Agarose 0.6%: 6 g of indubiose A37 (l'Industrie Biologique Française) dissolved in 100 ml of PBS by boiling for at least 10 min

Coated RBC: Prepare as described in Section V;B,1. Use as 1 : 4 (v/v) suspension in PBS.

Monoclonal antibody: If tissue culture supernatants are to be tested add one drop of 5% NaN_3, 1.2 *M* HEPES to 1 ml of spent medium.

Developing antibody: Anti-rat or mouse immunoglobulin antiserum. The developing antibody should not lyse coated RBC and should be titrated in a spot test procedure similar to the one to be used: excess causes inhibition of lysis.

Guinea pig complement (GPC'): Blood from normal adult guinea pigs is allowed to clot at 37° for 1 hr. Clarify the serum by centrifugation at 1500 *g* for 20 min at 4°. To 9 volumes of serum add 1 volume of 0.1 *M* EDTA in PBS and 3 volumes of packed RBC. Incubate for at least 1 hr at 4° with continuous mixing by inversion of the tube (about 1 inversion per second). Add 1 volume of $CaCl_2$, 0.1 *M* in PBS. Centrifuge for 10 min at 1500 *g* at 4°. The GPC' should be aliquoted and stored at −70° or, preferably, in liquid N_2. Complement activity is easily lost by freezing and thawing. Thawing is done at 37° with mixing.

Petri dishes: The procedures given below apply to plastic petri dishes 9 cm in diameter. Different sizes can be used, adjusting the reagent volumes. It is advisable, particularly if glass petri dishes or small plastic ones are to be used, to coat them by pouring a base of 7 ml of 1.5% agarose in PBS in each 9-cm dish. These can be stored inverted in a humid chamber at 4° for several weeks. Before use, a reticulate is drawn with a Magic Marker on the base of the dish, dividing it into approximately 20–30 identifiable areas.

Glass tubes: The convenient size to hold 2 ml of agarose is about 5 cm long, 10 mm in diameter, round-bottomed and rimless.

[26] H. S. Goodman, *Nature (London)* **190**, 269 (1961).
[27] T. Pearson, G. Galfre, A. Ziegler, and C. Milstein, *Eur. J. Immunol.* **7**, 684 (1977).
[28] N. K. Jerne and A. A. Nordin, *Science* **140**, 405 (1963).

Quick Mix Procedure

1. In a glass tube kept at 42° add 2 ml of 0.6% agarose, 100 μl of RBC, 200 μl of GPC', and 100 μl of developing antibody at an appropriate dilution.
2. Mix well by rotating the glass tube between the hands and pour immediately onto the petri dish to form an even layer. Let the agarose set for 5–10 min.
3. Spot onto the marked area of each petri dish 3–5 μl of the spent medium to be tested. Cover with the lid and incubate at 37° in a humid chamber. Lytic areas are generally evident after 1 hr, but longer incubations may be required. Lysis can best be seen against a dark background and with lateral illumination.

Two-Step Procedure

1. To 2 ml of 0.6% agarose kept in a glass tube at 42° add 100 μl of RBC.
2. Mix well by rotating the tube between the hands and pour to form an even layer on the petri dish. Set for 5–10 min.
3. Spot 3–5 μl of the supernatants to be tested. Allow the drops to dry, leaving the dish open, for 5 min. Cover and incubate at 37° in a humid chamber for 1 hr.
4. Pour into each dish 3 ml of a solution containing 10% GPC' and a titrated amount of developing antibody.
5. Incubate at 37° in a humid chamber. Lytic areas are generally evident at 1 hr, but it is advisable to incubate for at least 4–6 hr before giving results a negative score.

D. Assays Based on Biological Activity of Antigen

Antibodies can be recognized by their effect on the biological activity of an antigenic substance. The simplest way is to add individual culture supernatants to a biologically active preparation of antigen (e.g., an impure interferon preparation[29]). After a suitable period of incubation, a decrease in biological activity is taken as preliminary evidence for the

[29] D. S. Secher and D. C. Burke, *Nature (London)* **285**, 446 (1980).

presence of inhibitory antibody. On the other hand, the precipitation of antigen–antibody complexes can be effected by different procedures; for instance, by addition of carrier mouse or rat immunoglobulin and anti-mouse or rat immunoglobulin to equivalence. Alternatively, anti-rat or anti-mouse immunoglobulin or protein A attached to Sepharose can be used to absorb the antigen–antibody complexes.[30] The important aspect of these methods is that they afford an exquisite specificity of recognition without the need for antigen purification.

VI. Direct Detection of Antibody-Secreting Cells

Direct identification of antibody-producing cells relies on detecting the minute amount of antibody present on the cell surface or in the immediate vicinity of the cells immobilized on semisolid medium.

Antibody-secreting cells, under appropriate conditions, bind a certain amount of antigen. It has been possible to detect cells secreting specific antibody by attaching the antigen to fluorescent microspheres. A method has recently been described whereby cells in suspension are rendered fluorescent in this way and are automatically separated from the rest by a fluorescent-activated cell sorter.[31]

When cells are grown on a semisolid support of agar or agarose, the antibody secreted by the cells diffuses slowly, and methods have been developed to visualize it, either *in situ*[1,32] or by replica methods. The replica immunoadsorption method[33] is based on the adsorption of the antibody secreted by the clones onto nitrocellulose filters that have been precoated with antigen or anti-immunoglobulin. The filters are placed on the surface of the agarose containing the growing hybrid clones. After a suitable time, the filter is removed and the presence of localized areas containing specific antibody can be revealed by the binding of labeled antigen. For instance, a suspension of antigen-coupled erythrocytes is overlaid and the unbound erythrocytes washed away. Red spots delineate the sites at which antibody-forming clones are present in the agarose.

A. Plaque-Forming Clones

We describe here in detail a method[34] for the direct visualization of antibody-secreting clones based on the complement-dependent, localized

[30] T. Pearson and L. Anderson, *Anal. Biochem.* **101**, 377 (1980).
[31] R. Parks, V. M. Bryan, V. T. Oi, and L. A. Herzenberg, *Proc. Natl. Acad. Sci. U.S.A.* **76**, 1962 (1979).
[32] N. K. Jerne, C. Henry, A. A. Nordin, H. Fuji, A. M. C. Koros, and I. Lefkovits, *Transplant. Rev.* **18**, 130 (1974).
[33] J. Sharon, S. L. Morrison, and E. A. Kabat, *Proc. Natl. Acad. Sci. U.S.A.* **76**, 1420 (1979).
[34] C. Milstein and B. W. Wright, unpublished data, 1979.

lysis of antigen-coated red blood cells. The optical properties of sheep RBC allow easy visualization of local areas of lysis around antibody-secreting clones. The lysis of other types of target cells can also be used to localize antibody, but then live and dead target cells are visualized by vital strains.[35] The method has been applied to the detection and isolation of clones secreting antibodies to cell surface antigens.[36]

Materials

Agarose, 1.2% (w/v): The quality of the agarose is critical. It often has anticomplement activity. From this point of view, indubiose A37 is best, but we have been unsuccessful in using it for cloning (although it is best for overlays; see below). Some batches of LGT agarose (Marine Colloids, Inc.) are appropriate for both: preliminary tests, using it for spot tests (as described in Section V,C), are recommended for new batches. Agarose is suspended in tissue culture grade distilled water, autoclaved, and kept at 42°.

Concentrated FCS-DMM: 500 ml of 2 × DMM (Section II,A); 20 ml of penicillin-streptomycin, 5000 units/ml 200 ml of FCS; and 10 ml of 100 m*M* sodium pyruvate

Agarose, 0.5%: 1 volume of 1.2% agarose and 1.2 volume of concentrated FCS-DMM. Keep at 42°.

Cells: A vigorously growing culture should be used. Wash cells and prepare suspensions in 20% FCS-DMM containing appropriate cell dilutions (e.g., 1000, 5000, and 25,000 cells/ml). Keep at 37°.

Linbro plates: 6-dish Linbro plate (Flow Laboratories, Cat. No. FB-6-TC)

Coupled SRBC (1:4 in PBS), guinea pig complement suitably absorbed and developing second antibody (optional) are as described in Section V,C.

Preparations of Base Layers. Use at least one 6-dish plate for each culture to be cloned. The dishes should preferably be seeded with a feeder layer 24 hr in advance. Remove all the medium and apply 2 ml of 0.5% agarose to every dish. Set at 4° on a level surface.

Cloning. Take 150 μl of 0.5% agarose and add 135 μl of each cell suspension and 15 μl of coupled SRBC. Apply dropwise and as evenly as possible to the top of the cold agarose base layers. Prepare duplicates using 150 μl of 0.5% agarose and 150 μl of cell suspension, but no SRBC. Controls with 20% FCS-DMM substituting the cell suspension should also be carried out to test the stability of red cells in the absence of hybrids. Put plates back into refrigerator; keep level for about 15 min to

[35] H. Fuji, M. Zaleski, and F. Milgrom, *J. Immunol.* **106**, 56 (1971).
[36] P. Lake, E. A. Clark, M. Khorshidi, and G. M. Sunshine, *Eur. J. Immunol.* **9**, 875 (1979).

solidify top layer. Transfer to 37° in CO_2 incubators. Check for growth after 48 hr.

Revealing Clone Plaques

1. The cultures containing the coupled SRBC should be tested at about 48 hr. Densely growing cultures could result in complete lysis. Add to each well 0.3 ml of 10% FCS–DMM containing 10% GPC' (and developing antiglobulin antibody at the appropriate concentration when required). Replace plates in the incubator. Observe under dark field after 2 hr, 4 hr, and overnight. A stereomicroscope with low magnification is very useful for this purpose. Areas of lysis (plaques) should appear around antibody-producing clones. These are allowed to grow for 7–10 days before picking them.

2. The wells that do not contain red cells can be left for about 7 days, and clone plaques can be revealed by an overlay procedure.[1] Add to each well 0.3 ml of a suspension made up of 2 ml of 0.5% agarose, 0.2 ml of GPC' and 0.1 ml of coated SRBC. Addition of anti-mouse immunoglobulin antibody is also recommended. The amount to be used should first be titrated as it is inhibitory in excess. Lytic areas around clones usually appear within 2 hr at 37°.

Variations in the order of addition of the reagents can have dramatic effects on the results. For instance, the agarose overlay containing the red cells can be applied first. After incubation for 2 hr, a 0.3 ml solution containing the guinea pig complement and the second antibody is added. Lytic areas should appear on further incubation at 37°. This two-step method allows the monoclonal antibody to bind to the red cells before the antiglobulin reacts with it and is more sensitive. At first sight this appears less risky, but this is not so. The problem is much more complex, and in some cases rings of lysis are observed. This is because the essential requirement for lysis is the formation of an aggregate of monoclonal antibody and anti-immunoglobulin at the surface of the red cell. Excess of either of the two antibodies is inhibitory. The concentration of monoclonal antibody decreases as the distance from the clone increases. The rings of lysis appear at the point at which both antibodies are at equivalence.[37]

VII. Cloning

The timing of the cloning requires careful consideration. As a general rule it is best to clone as early as possible. Multiple clones in a single culture compete for growth and this, together with chromosome segregation, conspires against stability of expression (see Section VIII). However, hybrid lines are easier to clone after some time of active growth. It is possible to clone immediately after fusion (step 14, Section IV,B,1) without prior fractionation on Linbro plates. This is not recommended unless a method of direct detection of antibody-secreting clones is being used (Section VI).

[37] C. D. Wilde, Ph.D. Dissertation, Cambridge Univ. Library, 1979.

If the standard protocol in Fig. 3 is followed, supernatants from the microcultures at step 19 of Section IV,B,1 have been assayed. The cloning strategy somewhat depends on the number of independent positive cultures.

1. If only a few cultures are positive, it is worthwhile to subdivide them by limiting dilutions (see below), preferably in the presence of a feeder layer, and at the same time subject them to a cloning procedure.
2. If there are too many positive cultures to be conveniently handled in this way, duplicates should be prepared (to minimize the risk of accidental loss) and cell stocks be frozen in liquid N_2.

It is important at this stage to attempt to assess the interest of the different antibodies. This assessment must give priority to the use for which the monoclonal antibody is intended. There are two aspects of the antibody properties to be assessed. One relates to the antigenic recognition, and the other to the functional properties of the antibody. Antigenic recognition includes cross-reactive patterns, antigenic distribution on natural carriers, and fine specificity. Other functional properties include kinetic and thermodynamic parameters of antigen–antibody interactions, cytotoxicity, agglutination, effect of the antibody on the biological activity of antigens. In this analysis it must be kept in mind that, at this stage, supernatants may contain multiple antibody species.

When individual cultures are identified as of special interest they can be treated as in item 1 above. It may be that no special preference can be attached to individual cultures, in which case they are probably best left growing and more frozen stocks prepared. Instability and clonal competition will simplify the problem. Supernatants should be tested at regular intervals. Some cultures will gradually become negative, and the more resilient ones are those that will be easiest to clone and to handle.

If at any stage it is found that interesting clones have been lost, attempts to recover them can be made using the frozen stocks. In this event only stocks prepared well before the culture became negative should be used. It must be remembered that antibody can still be present when antibody-producing cells are no longer growing.

1. *Limiting Dilution Fractionation*

About 3×10^5 cells are transferred to the first cup of a 24-well Linbro plate containing a feeder layer. After thorough mixing a twofold dilution series is prepared over the first 12 cups (maximum dilution of about 600 cells in cup 12) or over all 24 cups (about one cell in cup 21 or 22 and none in cups 23 and 24). Part of the medium is changed every 4 or 5 days. Supernatants are collected and tested when the cultures approach confluence. The positive culture containing the minimum number of seeded cells can either be fractionated again as above or cloned in semisolid medium as soon as possible. Many variations on this basic protocol can be made. A common one is to use 96-well microtiter plates.

The positive cultures selected in this way should not be regarded as monoclonal. Correct cloning as described below should be performed at least once, and preferably twice.

2. *Cloning on Semisolid Supports*

Materials

2 × DMM–20% FCS: 100 ml of 2 × DMM from dry powder, 40 ml of FCS

1% agar: 1 g of Agar (Bacto Agar, Cat. No. 0140-01, Difco Laboratories) in 100 ml of tissue culture grade distilled water. Autoclave for 15–20 min. Keep at 42°.

0.5% agar: 1 volume of 1% agar, 1 volume of 2 × DMM–20% FCS. Keep at 42°. [If 10 × DMM is to be used, the 0.5% agar is better prepared by mixing 1 volume of 5% agar in water with 1 volume of 2 × DMM–20% FCS (prepared from the 10 × DMM), and 8 volumes of 20% FCS–DMM (prepared from the 1 × DMM).]

HAT or other additives as required

Petri dishes: 9 cm in diameter plastic, tissue culture grade (Sterilin, Cat. No. 304V).

Procedure. Pour into each petri dish (with or without a feeder layer) about 15 ml of 0.5% agar. Set for about 15 min at room temperature. Prepare several cell suspensions containing, for instance, 100, 500, 5000, and 50,000 cells/ml. Add 1 ml of 0.5% agar (at 42°) to 1 ml of each cell suspension (at room temperature). Mix by rotating the tube between the hands and pour immediately onto the agar base. Allow to set for at least 10 min at room temperature and incubate at 37° in a CO_2 incubator.

Clones can be picked at days 4–5 using a dissection microscope or, after 7–10 days, directly, and transferred to individual cups of a 24-well Linbro plate. It may be essential to have feeder cells in the cups, especially if small clones are picked. A clone should not be considered pure until it has been recovered from a plate grown at low density.

3. *Cloning by Limiting Dilution*

This is performed as described in Section VII,1 except that a single dilution is used so that, at most, only one cell is present in each microculture well. A fluorescent-activated cell sorter with a cloning attachment is very convenient for this purpose; otherwise we prefer cloning on semisolid supports.

VIII. Selection of Positive Clones

Positive clones growing on semisolid supports can be identified by direct detection methods (see Section VI). From those plates containing the lower number of growing clones, several (at least six) should be picked when they are about 100–1000 cells. They are then transferred for further growth (Section VII,2).

If direct detection of positives is not possible, clones are picked randomly. The number of clones to be transferred for further growth depends on the expected frequency of positive clones. An informed guess is based essentially on the history of the antibody titer of the hybrid culture. Large numbers of random clones should be picked when the antibody titer decreases with time of culture. Care should be taken to pick small clones as well as large ones. On the other hand, if consistent or increasing titers are being obtained over a period of weeks or months, a random collection of 24 clones is a convenient number. The picked clones are allowed to grow to confluence, and the supernatant is assayed for antibody activity.

If none of the picked clones is positive, the most probable explanation is the presence of more vigorous negative competing clone(s). These can be variant clones that have lost the ability to secrete complete immunoglobulin (Fig. 2). If this is the case, clones that are positive for immunoglobulin secretion can be detected by direct methods, even if present at very low frequencies. This is done by a reverse-plaque method.[38] The detailed protocol is as described in Section VI,A. The red cells are coated with anti-immunoglobulin antibody that has previously been purified by affinity chromatography. Alternatively the red cells can be coated with protein A.[39] An *in situ* precipitation method can also be used that does not rely on lysis of red cells.[40] The plaque-forming clones secrete immunoglobulin but not necessarily the specific antibody. Such clones should be randomly picked and assayed for specific antibody.[29]

A simpler means of concentrating the specific antibody-producing clone can also be attempted by limiting dilution fractionation as described

[38] G. Köhler, S. C. Howe, and C. Milstein, *Eur. J. Immunol.* 6, 292 (1976).
[39] E. Gronowicz, A. Coutinho, and F. Melchers, *Eur. J. Immunol.* 6, 588 (1976).
[40] P. Coffino and M. D. Scharff, *Proc. Natl. Acad. Sci. U.S.A.* 68, 219 (1971).

above. But if this is to be done it may be much better to use stocks of cells frozen at earlier stages. This can become a rather tiresome exercise involving hundreds of microcultures.[41] It is only justified when chasing particularly valuable specificities.

Sometimes cultures remain positive for long periods, yet the clones are invariably negative. This may be due to cloning conditions selecting against the positive clone. The best course of action is modification of cloning conditions and methods.

IX. Derivation of Variants

From the collection of positive clones derived from each hybrid culture, at least three random ones should be grown up and frozen stocks prepared as a precautionary measure. Parallel to this an analysis should be made to explore the possible presence of individual clones producing different antibodies and of clonal variants. Sometimes the assay of the antibody activity of the supernatant will give either a clue to or a strong indication of clonal differences. Otherwise a biochemical analysis of the antibody secreted is very useful. Several tests can be made but a simple one is based on labeling the secreted products with radioactive amino acids (see Section V,A,5) and subsequent electrophoretic analysis. The radioactive supernatants are directly analyzed by sodium dodecyl sulfate gel electrophoresis[42] in the presence of reducing agents. Preliminary dialysis is unnecessary. The antibody can also be analyzed by isoelectric focusing. Full details of the apparatus and procedures used to analyze a large number of samples are given by Secher *et al.*[43] Intact IgM penetrates the acrylamide gel only under special conditions.[44] Isoelectric focusing of separated chains is described by Köhler and Milstein.[1] It is essential to include control samples on the electrophoretic plates to permit easy interpretation of results. This analysis gives a description of the chain composition of the antibody and distinguishes the γ and μ classes of heavy chain. Depending on the choice of parental myeloma line used for fusions, segregants that have lost the expression of the myeloma chains may be detected, and stocks from these should be frozen separately.

On the basis of the above analysis, of the growth characteristics of each clone, and of the stability of the antibody titer of the confluent supernatants, individual clones are transferred to bottles for the preparation of frozen stocks. Larger amounts of antibody can now be prepared,

[41] A. F. Williams, G. Galfrè, and C. Milstein, *Cell* 12, 663 (1977).
[42] U. K. Laemmli and M. Faure, *J. Mol. Biol.* 80, 575 (1973).
[43] D. S. Secher, C. Milstein, and K. Adetugbo, *Immunol. Rev.* 36, 51 (1977).
[44] A. Ziegler and H. Hengartner, *Eur. J. Immunol.* 7, 690 (1977).

but at the same time we consider it to be essential to reclone the line to ensure monoclonality and to achieve better stability of production. Even the best cloning technique cannot totally exclude the possibility of cross-contamination. A second cloning step performed at low cell density provides a fail-safe device.

It is better to use cultures that have been growing for a certain time for the second cloning. This is because for several months after fusion dividing hybrids tend to lose chromosomes and to attain a more stable genotype. It is therefore convenient to allow a certain amount of drift to facilitate the selection of a subclone that will have the best stability properties and the desired chain composition. The derivation of subclones follows the protocol described in Section VII. The number of subclones to be collected is based on the considerations discussed in Section VIII, except that when dealing with stable lines fewer subclones can be picked. However, if chain loss variants are sought many more subclones must be screened.

Procedure for the Derivation of Chain Loss Variants

1. It is preferable to use a culture that has not been recloned and that has been in continuous growth for a reasonable period (a month or longer).
2. Prepare clones as described in Section VII,2.
3. With a Pasteur pipette suck up a plug of agar containing a single large (at least 1000 cells) clone and blow the agar plug into a well of a microtiter plate containing 150 μl of incorporation medium (Section V,A,5). A few cells remain in the pipette, and these are carefully washed into a well of a second microtiter plate containing 150 μl of 20% FCS–DMM, with or without a feeder layer.
4. Repeat this procedure with at least 48 clones.
5. Put both plates in a humid CO_2 incubator.
6. After at least 16 hr of incubation, centrifuge the plate containing the radioactive samples for 5 min at 400 g. Transfer the supernatant to the empty wells of the plate or to another plate.
7. Analyze the radioactive supernatant by the electrophoretic method of choice.
8. If variants are identified, the culture contained in the replica plate is transferred into a larger culture dish.
9. Recloning of the selected variant should be performed as soon as possible because of the high risk of cross-contamination introduced by the requirement of large clones at step 3.

A procedure based on anti-idiotypic antiserum has been described.[45]

[45] T. Springer, *J. Immunol. Methods* (in press).

X. Feeders

There is no doubt that the presence of a feeder layer increases the ability of cultured cells to grow at very low densities. The use of feeders is therefore essential for isolating hybrid clones that are otherwise difficult to grow. It increases the yield of viable hybrids after the fusion step and is strongly recommended in the fusion protocol (Section IV,B,1) and is essential for the protocol of Section IV,B,2. It is also essential when cloning by limiting dilution. However the indiscriminate use of feeders introduces an often unnecessary complication. Furthermore, as the final aim of the overall protocol is the preparation of cloned hybrid lines that will grow vigorously in the least demanding culture media, we prefer to avoid the use of feeders as soon as this is possible.

For the fusion itself the simplest, although not necessarily the best, feeder is the same cell as used for the fusion. Different workers have recommended other normal cells, notably thymocytes and macrophages. It is objectionable and often less convenient to use cells from specially sacrificed animals. Feeders made from irradiated fibroblasts are a good alternative. Many different fibroblast lines can be used, and we have obtained reasonable results with the 3T3 mouse line obtainable from most tissue culture collections and suppliers.

Procedure. A large culture of fibroblasts is harvested in the logarithmic phase of growth, washed by centrifugation and irradiated with about 10,000 rad. After irradiation the cells are resuspended in freezing medium and frozen (see Section XI) in aliquots of about 5×10^5 cells/0.5 ml. Titration and control of each batch prepared is necessary. A vial is thawed, and from this twofold dilution cultures are prepared in a 24-well Linbro plate. After 3–4 days of culture, the well giving a 50% confluent monolayer is used to calculate the number of wells that can be prepared from each frozen vial. The dilution plate is kept for a further 7–10 days to check that no further growth is evident.

XI. Freezing of Cells

Many methods for freezing viable cells have been described, and some rely on fairly sophisticated apparatus to provide programmed temperature decrease. The method we will describe does not rank high in terms of recovery of viable cells, but it is extremely simple and ideally suited to the specific needs of derivation of hybrid myelomas. We find it very reliable provided that the cell stock used for freezing is in full logarithmic growth.

Freezing Procedure. About 10^6 to 10^7 cells are pelleted by centrifugation at 400 *g* at 4° for 7 min. The supernatant is removed, and the pellet is resuspended in 0.5 ml of freezing medium (9 parts FCS, 1 part dimethyl sulfoxide) at 4°. The suspension is transferred to a freezing vial (Sterilin, Cat. No. 506), and this is placed in a small insulating box (1 cm thick expanded polystyrene is adequate) and put at −70° for at least 20 hr. The vial is then transferred directly to liquid N_2.

Thawing Procedure. Thaw the vials as quickly as possible in a 37° water bath. When thawing is nearly complete, transfer the cell suspension to a 10-ml centrifuge tube in an ice bath. Slowly add 10 ml of cold 10% FCS–DMM, mixing carefully. Centrifuge at 400 *g* at 4° for 7 min. Resuspend the cells in about 5 ml of fresh medium, and transfer to a small tissue culture flask. It is better, but more laborious, to resuspend the cells in 2 ml and prepare a series of twofold dilution cultures in Linbro plates, with or without feeders.

XII. Large-Scale Production of Monoclonal Antibody

Large amounts of monoclonal antibody can be produced either by culturing cells *in vitro* or growing them as tumors *in vivo*. The monoclonal antibody is secreted and is accumulated in the spent medium of the cultured cells and in the serum and body fluids of the tumor-bearing animals. The two methods of production are complementary, as both have advantages and disadvantages. The concentration of monoclonal antibody in the spent medium is of the order of 10 μg/ml but can be increased to perhaps 50 μg/ml or even 100 μg/ml. The concentration of monoclonal antibody in the serum of a tumor-bearing animal is often about 10 mg/ml and may reach 3 or 4 times that value. The animal serum is therefore usually 1000 times more concentrated. But this is not always so. It has been observed that certain macroglobulins never reach high concentrations in the serum. The reasons for this are not clear, but it seems that some macroglobulins have a higher catabolic rate, preventing their accumulation in the serum. In one example the anti-blood group A activity of a monoclonal antibody taken from the serum was of a lower quality than the equivalent product taken from tissue culture. This was interpreted as being due to partial proteolytic degradation.[46]

The protein impurities present in the spent medium can largely be controlled because most come as components of medium. In particular the monoclonal antibody is the only immunoglobulin of rat (or mouse) origin in the spent medium. In contrast, serum from tumor-bearing animals always contains immunoglobulin impurities that are of the same species. Although such animals have a severe depletion of their normal

[46] D. Voak and C. Milstein, unpublished data, 1979.

immunoglobulin components, the antibody is not likely to be much better than 90% of the pure monoclonal variety. Tissue culture material is therefore intrinsically better as a source of monoclonal antibody. It is to be preferred when concentration is well above that required.

The high concentration of monoclonal antibody in the fluids of tumor-bearing animals makes them better for the preparation of chemically purified antibody. The purification protocol has to be adapted to each individual case, depending mainly on the antibody class. Usually a 50% ammonium sulfate precipitate gives better than 50% pure monoclonal antibody. Further purifications (for instance DEAE-column chromatography) are widely discussed in the literature. In the long run, even large-scale preparations of pure monoclonal antibody may use spent medium from cultured cells as a more humane and better controlled source. But this will depend on the technological development of large-scale cell culture methods.

A. Production in Culture

Before large-scale growth it is advisable to adapt the chosen clone to medium containing a low percentage of serum. This is usually achieved by feeding a vigorously growing culture with 5% FCS–DMM.

For Small Quantities. Transfer 20 ml of cells from above to a tissue culture bottle (800 ml, Flask Nunclon-Delta, Cat. No. N-1475, Nunc, Denmark) and dilute to about 50 ml with 2.5% FCS–DMM. Gas with 10% CO_2–90% air. Close bottle tightly and keep it in a dry incubator at 37°. After 1–2 days add a further 150 ml of 2.5% FCS–DMM and let the culture grow for a further 2 days or more until it has been in stationary phase at least one day. Collect the supernatant by centrifugation.

For Medium Quantities. Transfer 30 ml of a vigorously growing culture into a roller flask (850 cm^2 Roller Bottles, Falcon, No. 3027) and add 70 ml of 2.5% FCS–DMM. Gas as above. Close bottle tightly and keep it standing at 37° in a dry incubator. After 1–2 days add 700 ml of 2.5% FCS–DMM, close tightly, and keep rolling (1 rpm) at 37°. Open the bottle daily for 5–10 min in a sterile hood to allow gas exchange. Harvest the supernatant at least 1 day after cells have reached the stationary phase of growth.

For Larger Quantities. Transfer 200 ml of a vigorously growing culture to a 5-liter spinner (Fig. 1), dilute it with 200 ml of 5% FCS–DMM, and check for growth after 24 hr. If growth is vigorous, start diluting the culture with 2.5% FCS–DMM at a rate that will keep the culture in logarithmic growth. When the spinner is full, allow it to achieve stationary phase, leave it for a further 1–2 days, and harvest. Checks of antibody titers can be used as an indicator of the best harvesting time.

If even larger volumes are required a series of spinners can be organized in such a way that one spinner is kept permanently in logarithmic growth with 5% FCS–DMM and the others are used to dilute with 2.5% FCS–DMM.

Serumless Preparations. For synthetic media that do not include serum some formulations have been proposed.[47] But ordinary DMM (with no serum) can also be used. Prepare a vigorously growing culture in 5% FCS–DMM. Centrifuge cells and resuspend at a density of 1 to 4 × 10^6 cells/ml in DMM. Gas the flask. Incubate at 37° for 24–48 hr. Harvest.

Concentration of Antibody from Spent Medium

Spent medium can be concentrated using ultrafiltration devices (e.g., Minicon Concentrator, B15, Amicon Corporation). For larger volumes we prefer the following procedure.

Add solid $(NH_4)_2SO_4$ with gentle stirring to 50% saturation. Allow to equilibrate for at least 30 min. Centrifuge. Dissolve the precipitate with PBS (or alternative saline solution) using a volume of about 1/100 of the original spent medium. Dialyze against the chosen saline solution and clarify by centrifugation. The procedure is best carried out at 4°. Some monoclonal antibodies may be unstable to this treatment.

B. Production in Animals

Tumors can be derived as either solid or ascitic. Solid tumors are somewhat easier to derive and to manage, but the yield of antibody is usually higher from ascitic fluid. Animals should be histocompatible with the hybrid clone to be injected. For instance, a clone made with myeloma X63 (BALB/c origin) and spleen cells from a C3H mouse should be injected into (BALB/c × C3H) F_1 hybrids. Partial mismatching is sometimes acceptable but may require immunosuppressive treatment. In any event partial immunosuppressive treatment is often recommended for faster tumor growth. In some cases this is essential even with fully histocompatible combinations, possibly owing to somatic drift of tumor antigens. Adequate immunosuppression is usually achieved with a relatively low X-ray irradiation dose (say 500 rads) and/or an injection of about 0.5 mg of cyclophosphamide/20 g animal weight 24 hr before tumor transplantation. The use of drastic immunosuppression or immunodeficient strains of animals (e.g., nude mice) has been recommended for the growth of totally histoincompatible tumors, such as clones derived from a mouse myeloma and spleen cells from rats. We did not find that this procedure

[47] N. N. Iscove and F. Melchers, *J. Exp. Med.* 147, 923 (1978).

yielded better material than we could prepare by concentrating spent medium.

Solid Tumors. Cells taken from a vigorously growing culture are centrifuged and resuspended to a cell density of about 1 to 3 × 10^7/ml in 5% FCS–DMM. Animals are inoculated subcutaneously in the center of the back, not too near the neck, and high up in each flank near the spine. Mice are given 0.2 ml of cell suspension in each site and rats about twice as much, using a somewhat higher cell density. Freshly excised tumors can be used for transplanting in other animals. Tumors of a good size are sliced (discarding necrotic parts) in a petri dish containing 10 ml of Earle's balanced salt solution. A cell suspension is prepared with a loose-fitting homogenizer. The preparation can be used to inoculate about 10 other animals.

Ascitic Tumors. Before the induction of tumors mice should be inoculated intraperitoneally with 0.5 ml of pristane (tetramethylpentadecane). After 1–9 weeks about 10^7 cells are suspended in 0.5 ml of medium and injected intraperitoneally. As soon as ascitic fluid accumulates (usually about 10 days after inoculation), it is removed by "tapping" the mouse. For this a hypodermic needle (size 19, 1-inch or 20 G 1½ inch 40/9) is inserted in the abdominal area close to the surface. The liquid that drips off is collected in a suitable container. This first "tap" does not usually contain a high concentration of antibody. Further tapping should be carried out every 1–3 days. It is possible to repeat the operation perhaps 10 times without sacrificing the animal.

Control of Production

It is important to monitor the concentration of the monoclonal antibody in the serum/ascites at every tumor passage. It is not uncommon to find that on continuous passage tumors lose the capacity to produce the antibody. This is most probably due to negative variants with increased malignancy overgrowing the original positive cells. The monitoring can be done by determining the antibody titer using the most convenient assay. Alternatively, direct determination of myeloma protein concentration can be made by conventional methods. Electrophoresis on cellulose acetate strips, as routinely performed for blood samples in hospital laboratories, gives a very fast visual estimation of the concentration as well as of the mobility characteristics of the monoclonal antibody. This is a reassuring chemical check. Accidental mix-ups may not be detectable by specific antibody tests if the products are directed against a common target.

If the production of antibody declines on continuous passage, new tumors should be induced from frozen stock. If the production goes negative after only a few passages, it may be necessary to prepare a more stable clone, using the tumor cells for recloning.

C. Storage

Generally speaking monoclonal antibody can be stored as conventional antisera. Sterile samples can usually be stored for reasonable periods at 4°. Addition of preservatives such as NaN_3 at 0.1% is common. For very long periods it is probably better to store at −20°, but freezing and thawing should be avoided whenever possible, especially when dealing with IgM antibodies.

There is a critical difference between monoclonal and conventional antibodies as regards stability. Conventional antisera contain many different monoclonal antibodies, each with different stability. If only some are sensitive to a particular treatment, the activity of the preparation may not be seriously impaired by that treatment, even when done repeatedly. For instance, one out of two monoclonal antibodies to antigen X may be totally destroyed during freeze-drying, but the activity of their mixture will only decrease to 50% of the original. It is advisable to test the stability of a given monoclonal antibody to any particular treatment before committing a large batch.

XIII. Unusual Properties of Monoclonal Antibodies

When compared with ordinary antisera, monoclonal antibodies are likely to display unusual serological features. The most obvious differences arise from synergistic efects. For instance, unless the antigen contains multiple identical subunits, the monoclonal antibodies are unlikely to give precipitating reactions because no three-dimensional lattices are likely to be produced.[4]

Cytotoxicity reactions are affected not only by the class of the monoclonal antibody, but also by the local distribution of the determinants on the cell surface. This local concentration can be increased dramatically by multiple antibodies recognizing the same antigen. For instance, in a case of two different monoclonal antibodies recognizing histocompatibility antigens, neither alone is cytotoxic, but the mixture of the two is strongly so. Although these types of synergistic effects can be very confusing, they can also become very useful tools—for example, to reveal cells secreting a "nonlytic" antibody, using red cells pretreated with a previously isolated monoclonal antibody.[24]

Cooperative effects are also likely to be among the reasons why monoclonal antibodies are often less good agglutinators than the conventional (polyclonal) antisera.[22] But other facts are likely to complicate the problem. For instance, the indirect hemagglutination by a monoclonal anti-IgG was found to be very different when sheep red cells were coated with two different monoclonal anti-sheep red cells. It was *negative* when the cells were coated with Sp2 (a monoclonal antibody recognizing a *high*

density determinant) and *positive* when coating was with Sp3 (recognizing a *low* density determinant).[6] The precise reasons for the effect may involve structural features, but also the precise ratios of molecular species become critical because the reagents are monoclonal. Complex mixtures are to a certain extent self-correcting, because different antibody species present at different concentrations can act independently of each other. The use of preparations containing mixed molecules secreted by HLGK or HLK clones (Fig. 2) introduces further complications.

Precipitation analysis of labeled monoclonal antibodies mixed with polyvalent antisera is a method that is likely to be used extensively. It was observed that under these conditions the labeled monoclonal antibodies were able to diffuse through the precipitin lines to which they bind and coprecipitate with another line. This is contrary to the old assumption that precipitin lines act as diffusion barriers. One monoclonal antibody in excess may not be able to dissolve the precipitate and diffuse through it to bind to other precipitin lines containing the same determinant on a different molecular species.[22]

The fine specificity of monoclonal antibodies is a great asset but should be used with caution. Negative results with a monoclonal antibody do not prove absence of the antigen itself. Changes in the environment of the antigenic determinant, or of the way the antigen is presented, could alter results. On the other hand, reaction with a monoclonal antibody could, at least in theory, occur through recognition of more than a single antigenic structure. More commonly the same antigenic determinants could be expressed in different molecular species—e.g., carbohydrate moieties or structural features in evolutionarily related proteins.

[2] Production of Antisera with Small Doses of Immunogen: Multiple Intradermal Injections

By JUDITH L. VAITUKAITIS

A wide variety of immunization techniques has been used to generate specific antisera in laboratory animals. Those techniques incorporate a variety of injection routes, vehicles, and frequencies of injection into appropriate laboratory animals. Moreover, the concentrations of immunogen have ranged from gram to milligram concentrations. With the advent of more sophisticated isolation techniques, as well as the capacity readily to synthesize polypeptides, successful immunization with small amounts of immunogen has become imperative. Consequently, an approach to

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[1] J. L. Vaitukaitis, J. B. F
33, 988 (1971).
[2] J. L. Vaitukaitis and G.
[3] H. N. Eisen and G. W.